Mitochondrial dysfunction and axon degeneration in progressive multiple sclerosis

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Abstract

The neuron is the target of inflammatory demyelinating processes in multiple sclerosis (MS). In progressive MS, however, there is a gathering body of evidence indicating that molecular changes converge on mitochondria within neuronal cell bodies. The most reproducible change relates to mitochondrial respiratory chain complex deficiency, which compromises the capacity of neurons to generate ATP. The resulting energy failure state is coupled with an increase in demand for energy by the demyelinated axon, being particularly relevant to the long tracts such as corticospinal tracts with long projection axons. Recent work in our laboratory and that of our collaborators indicates the limited reflection of the mitochondria changes within neurons in experimental disease models. The mitochondrial changes within neuronal compartments are likely to offer novel targets for the improvement in neuronal function in patients with progressive MS.

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Introduction

Progressive multiple sclerosis (MS) is a chronic inflammatory demyelinating disorder with ongoing neurodegeneration.(Stadelmann 2011) Inflammation is a potent inducer of damage to neurons, particularly axons and synapses in MS. Furthermore, the restoration of myelin to denuded axons (remyelination) is limited in progressive MS, which leaves the naked axons much more vulnerable to additional insults. There is an overall tendency for inflammation to become less prominent with increasing disease duration, although with case-to-case variability even at the end stage of the disease.(Mahad et al. 2015) In contrast to inflammation, the lesion burden or the extent of demyelination gradually increases partly due to lack of remyelination as well as ongoing and repeated inflammatory demyelination. The cumulative impact of inflammation and chronic demyelination leads to the loss of CNS tissue, as reflected by brain and spinal cord atrophy. Neuropathological studies indicate extensive loss of axons particularly in the spinal cord long tracts and synapses in the grey matter (>60-70% loss).(Bjartmar et al. 2003; Jurgens et al. 2016) In contrast, the extent of neuronal cell body loss in the cortical grey matter is marginal, with 15-20% loss, except in cases with so called follicle-like B cell infiltrated in the meninges.(Magliozzi et al. 2010) The existence of neuronal cell bodies at the end stage of progressive MS in autopsy tissue offers the opportunity to study their content and gain insight into how the neurons may play a role in and contribute to the neurodegenerative process of progressive MS.

We discuss the molecular, proteomic, biochemical and dynamic mitochondrial changes that are intrinsic to neurons, (Mahad et al. 2015) and how these molecular changes lead to an energy failure state through an increase in demand for ATP and a decrease in capacity to produce ATP by mitochondria.

Molecular changes within the neuronal cell bodies in progressive MS

It is abundantly clear that the mitochondria within neuronal cell bodies are damaged in progressive MS (Figure 1). All these molecular changes, discussed in more detail below, converge on mitochondrial function and transport. The molecular changes within neurons in progressive MS impact the mitochondrial respiratory chain complexes and hence the ability to produce ATP by oxidative phosphorylation.

Mitochondrial respiratory chain complexes in neuronal cell bodies

The first detailed study of upper motor neurons from non-demyelinated motor cortex was reported by Dutta et al just over a decade ago.(Dutta et al. 2006) These authors subjected well-preserved grey matter tissue from rapid autopsy cases to microarrays in an unbiased manner and found the majority of the most significant molecular changes to be related to the mitochondrial respiratory chain complex subunits. They investigated nuclear DNA encoded transcripts and found 26 transcripts of mitochondrial respiratory chain complexes to have decreased significantly. This decrease in nuclear DNA encoded mitochondrial transcripts was neuron-specific and affected the majority of upper motor neurons. Furthermore, the transcript changes led to mitochondrial respiratory chain complex I and complex III deficiency. Notably, the mitochondrial respiratory chain deficient neurons were found in non-demyelinated motor cortex, which suggests a role for pathological processes such as demyelination of the axon in the white matter in the generation of neuronal mitochondrial defects in MS.

Since the publication of the above study in 2006, the decrease in nuclear DNA encoded mitochondrial respiratory chain transcripts in non-demyelinated cortex has been observed by others.(Broadwater et al. 2011; Witte et al. 2013) An independent group identified the transcripts of mitochondrial respiratory chain complexes to have significantly decreased in both the cingulate gyrus and frontal cortex.(Witte et al. 2013) Furthermore, authors of this study identified a significant decrease (quarter to one third) in mRNA level of peroxisome proliferator-activated receptor (PPAR)-gamma coactivator 1-alpha (PGC-1alpha), a master regulator of metabolism and mitochondrial function. The decrease in PGC-1alpha was evident in the deeper cortical layers (layers IV-VI). The mitochondrial respiratory chain complex deficiency in non-demyelinated motor cortex was associated with a decrease in components of GABAergic neurotransmission and loss of inhibitory interneuron processes as well as a decrease in mitochondrial anti-oxidants.(Dutta et al. 2006; Witte et al. 2013) Broadwater and colleagues undertook a different experimental approach to investigate mitochondria in the non-demyelinated cortex in progressive MS cases.(Broadwater et al. 2011) Using mass spectroscopy they identified proteomic changes relating to the mitochondrial respiratory chain complexes in the normal appearing motor cortex in progressive MS. A significant decrease in mitochondrial respiratory chain complex IV subunit V was confirmed by western blots. Furthermore, another study indicated a number of nuclear DNA encoded and mitochondrial DNA encoded transcripts to have decreased in acute white matter MS lesions.(Fischer et al. 2012)

Our study undertook a single cell approach and interrogated mitochondrial DNA and identified neurons in the deeper cortical layers that lacked complex IV and contained complex II, which in the literature are termed as respiratory deficient neurons.(Campbell et al. 2011) Laser micro-dissection of these respiratory deficient neurons and detailed analysis of mitochondrial DNA extracted from the captured cell identified clonally expanded mitochondrial DNA deletions at high heteroplasmy level. Heteroplasmy is when mitochondrial DNA mutation(s) co-exist with wild type genomes in a single cell.(Larsson 2010) The proportion of mitochondrial DNA mutations to total mitochondrial DNA content in a single cell designates the heteroplasmy level.(Larsson 2010) The process that leads to the increase in the copy number of mutant mitochondrial genomes in a single cell is termed clonal expansion of mitochondrial DNA mutation. Clonal expansion of mitochondrial DNA is well recognized in a number of neurodegenerative conditions, although the mechanism is not well-understood.(Larsson 2010) Judging by the current understanding of the time course of clonal expansion of mitochondrial DNA deletion in single cells, we expect it to occur over years in progressive MS. These respiratory deficient neurons, also reported in a number of classical neurodegenerative disorders, were more prevalent in non-demyelinated motor cortex and were not directly associated with microglia.(Campbell et al. 2011) These observations widen the possible causes and indicate an irreversible respiratory deficiency within neurons in progressive MS.

We then investigated the choroid plexus and skeletal muscle, which contain metabolically highly active post-mitotic cells, to extend the study of respiratory deficiency and mitochondrial DNA deletions in progressive MS to non-neuronal cells. Respiratory deficient choroid plexus epithelial cells were much more frequent in progressive MS cases compared with controls, Alzheimer's disease and Parkinson's disease.(Campbell et al. 2012) Again, we detected high heteroplasmy level of clonally expanded mitochondrial DNA deletions in the respiratory chain deficient cells. In contrast, we did not detect a significant change in the extent of respiratory deficient muscle fibres in progressive MS compared with age-matched controls.(Campbell et al. 2013) These findings suggest that the mitochondrial changes observed within neuronal cell bodies in progressive MS are likely to have been induced within the CNS, rather than representative of a multi-organ disorder with an inherited aetiology.

The cause(s) of these molecular changes intrinsic to neurons in progressive MS is not known, whether this is a primary effect or a secondary phenomenon following the loss of functional connectivity due to the loss of synapses and transection of the long projecting axon. In terms of the mitochondrial DNA deletions, the molecular events encompass a likely active or a positive selection phenomenon and the resulting mitochondrial respiratory chain complex deficiency is irreversible. Further studies are needed to understand both the cause of the neuronal molecular changes and the consequences of the neuronal mitochondrial respiratory chain deficiency,

Mitochondrial transport machinery within neurons in progressive MS

Beside the studies that found mitochondrial respiratory chain complex deficiency, other independent groups have identified changes that impact on the transport,of mitochondria from the cell body to the axon.

A study that investigated histone deacetylase 1 (HDAC1), which is an enzyme that is found within the nucleus and represses nuclear DNA transcription, detected aberrantly located enzyme in the cytoplasm and degenerated axons in MS autopsy tissue as well as in an in vivo experimental system with cuprizone induced demyelination.(Kim et al. 2010) This nuclear export of HDAC1 was neuron specific, related to the increase in calcium entry into the neuron and occurred prior to the damage to neuritis. The cytoplasmic HDAC1 formed protein complexes with kinesins (KIF2A and KIF5), which are motor proteins involved in anterograde transport of protein complexes, mRNA and membranous organelles, such as mitochondria, in axons. This interaction between cytoplasmic HDAC1 and kinesins, following exposure of neurons to glutamate and TNFalpha, impaired the anterograde transport of mitochondria in the axon. The inhibition of the nuclear export of HDAC1 using pharmacological agents prevented the damage to neurites. The impaired mitochondrial transport within axons in the context of inflammation was confirmed in experimental autoimmune encephalomyelitis (EAE) by another group.(Sorbara et al. 2014) Both anterograde and retrograde transport of mitochondria within axons was significantly decreased by a slower speed and more frequent stops. The transport deficits in EAE were rescued by methylprednisolone and redox scavengers. Further evidence of impaired anterograde transport of mitochondria in progressive MS is indicated by the significant decrease in motor proteins, called kinesins (KIF5A, KIF21B and KIF1B), within neurons.(Hares et al. 2013)

The impaired anterograde transport of mitochondria in neurons compromises the ability of the neuronal cell body to replenish the axon with new mitochondria. Whether agents, such as glucocorticoids, redox scavengers and anti-inflammatory drugs, that improve mitochondrial transport protect axons in progressive MS needs to be assessed in future studies. Furthermore, whether the manipulation of mitochondria protects demyelinated axons requires further investigation in pre-clinical systems that model the neuronal mitochondrial changes that are described in progressive MS.

Mitochondrial changes within demyelinated axons in MS

The energy demands and the distribution of mitochondria in the myelinated axon

A number of studies carried out over the past decade has shown evidence of mitochondrial changes within the demyelinated axon. To understand the significance of these changes, it is important to recognise the role mitochondria have to play in the myelinated axon within the CNS. Directed by the requirement for energy, the precise location of mitochondria within the axoplasm is crucial to axonal function.

The major advantage of myelination of axons is the saltatory conduction of action potentials which results in their fast propagation along the nerve. Recent evidence also suggests that myelinating cells of the CNS, the oligodendrocytes, supply axonal mitochondria with the metabolites required for them to efficiently perform oxidative phosphorylation to produce ATP, the cellular energy currency.(Funfschilling et al. 2012) Within the myelinated axoplasm, mitochondria are far from uniform, differing in morphology and velocity most often depending on their location, whether at the node of Ranvier, paranode, juxtanode or internode where changes in calcium levels and energy demand are different. It has been reported that the highly energy demanding Na^+/K^+ ATPase is richly distributed along the internode and juxtaparanode axonal membrane whilst absent from the nodal space and paranode.(Young et al. 2008) These changes are reflected in the distribution of mitochondria which varies dramatically along the axon. A third of nodes of Ranvier in mouse myelinated axons contain no mitochondria whilst the majority of the remaining nodes contain only a single mitochondria.(Ohno et al. 2011) Both the length and volume of mitochondria within the node and paranode are significantly decreased compared to their counterparts in the juxtaparanode and internode. In these regions, where large stationary mitochondrial networks are found, the ratio of mitochondrial to axonal volume is significantly increased. The variable distribution of the mitochondria is therefore likely to result from the changeable energy demands along the axon. It has also been hypothesized that axonal Ca^{2+} is

responsible for the distribution of mitochondria along the axon. Peripheral nervous system (PNS) axons show a marked increase in calcium signaling at the node of Ranvier,(Zhang et al. 2010) where mitochondria are highly enriched whilst *in vitro* evidence also suggests similar role of axoplasmic Ca^{2+} in the CNS.(Ohno et al. 2011) The mechanism for this may be an interaction of Ca^{2+} with the microtubule motor kinesin-1 responsible for anterograde transport of mitochondria along the axon.(Saotome et al. 2008) Whilst only shown in the peripheral nervous system (PNS) to date, artificial stimulation of the Na^{+}/K^{+} ATPase has also been shown to enhance retardation of mitochondrial velocity.(Zhang et al. 2010) Experimentally blocking action potentials results in a decrease in mitochondrial movement, whilst enhancing neuronal firing increases the mitochondrial movement, which suggests a likely role for axonal electrical conductivity in mitochondrial distribution.

Many of these factors become increasingly important, as dramatic axonal metabolic changes in the event of demyelination lead to important modifications in mitochondrial dynamics.

The changing energy demands of the demyelinated axon

Unmyelinated axons (axons that are never myelinated) provide a useful guide as to the prediction of mitochondrial changes one might observe in the demyelinated axon.(Bristow et al. 2002) Studies of the unmyelinated axons of the lamina cribrosa (site where axons exit the eye posteriorly to join the optic nerve) have provided such information where complex IV activity has been investigated. Complex IV is the terminal subunit in the electron transport chain and consumes 90% of cellular oxygen. The unmyelinated segment of the lamina cribrosa was found to have increased complex IV activity compared to its myelinated counterpart, an indicator of increased energy demand, highlighted by the uniform distribution of certain isoforms of Na channels (Na_v 1.1 and Na_v1.6) along the unmyelinated segment.(Bristow et al. 2002; Balaratnasingam et al. 2009) This is in contrast to myelinated axons where Na channels are specifically localized to the nodes of Ranvier within the CNS. The early redistribution of Na channels along the denuded axonal segment has been shown to be a consistent feature of demyelinated axons, which may allow the continuation of action potentials and in the context of MS, recovery of clinical function.(Craner et al. 2004b) The increase of complex IV activity has also been noted in animal models of dysmyelination (abnormal myelination, where the myelin sheaths are relatively thin, compared with normally myelinated axons) resulting from the loss of myelin basic protein and overexpression of proteolipid protein, respectively.(Andrews et al. 2006; Hogan et al. 2009) In both models, an

increase of axonal mitochondrial content was observed. It may be that the increase in axonal complex IV plays an important role, highlighted by the fact that in an animal model of only partial demyelination, caused by the hemizygous overexpression of PLP, an increase of mitochondrial content *without* complex IV activity was associated with axonal degeneration.

An increase in axonal mitochondrial content is a consistent feature of animal models of demyelination. Our recent studies (unpublished) show that this encompasses the classic modes of demyelination including lysolecithin, lipopolysaccharide, cuprizone, and various forms of EAE. It is now established that this is a feature of morphologically intact, large caliber axons in demyelinated lesions of MS (Figure 2). An increase in the axon-specific mitochondrial docking protein, syntaphilin, suggests that these mitochondria are likely to be stationary.(Mahad et al. 2009) *In vitro* evidence has provided further details of these changes including an increased size of stationary mitochondria in demyelinated axons, induced by lysolecithin and velocity of motile mitochondria.(Kiryu-Seo et al. 2010) It is perhaps surprising to note that the actual number of stationary mitochondria did not change, rather it is hypothesized that newly formed mitochondria fuse with those already at stationary sites along the axon. All of these changes were found to be reversed, *in vitro*, upon remyelination. This may not be entirely the case *in vivo* where axonal mitochondrial content does not return to baseline, intriguingly suggesting that remyelinated axons have different energy demands compared to myelinated axons, *in vivo*.(Zambonin et al. 2011)

It has been a matter of debate as to whether these mitochondrial changes in response to demyelination represent a compensatory or pathological phenomenon. Evidence does suggest, at least in the short-term, that these changes are essential for survival of the axon. Histological evidence from MS lesions indicates that axons that have morphological signs of degeneration and transport block do not show any increase in mitochondrial content.(Mahad et al. 2009) When the mitochondrial response to demyelination in axons is blocked, evidence for increased axon stress is observed and the experimental loss of syntaphilin, results in an increase of axonal degeneration.(Kiryu-Seo et al. 2010; Ohno et al. 2014) In the long-term however, recent evidence points toward a potential detrimental outcome for the accumulation of mitochondria in demyelinated axons. When syntaphilin was knocked out in the Shiverer dysmyelinated model an improved clinical outcome was noted with less axon degeneration. Thus, the degradation of unhealthy mitochondria, which can produce harmful ROS over a prolonged period of time, is likely to be important for the survival of demyelinating axons.(Joshi et al. 2015)

Axon degeneration in progressive MS - a specific role for complex IV activity?

Axon degeneration is a significant pathological hallmark of MS, its importance highlighted by the hypothesis that axon loss is the crucial factor in the development of progressive MS from relapsing-remitting disease.(Bjartmar et al. 2003) Inflammation is a key cause of axon degeneration particularly during the initial stages of disease. Anti-inflammatory treatments, effective during the acute stage of disease are rendered redundant in the progressive stage of disease, the reasons for which remain unresolved. Chronic demyelinated lesions persist for decades and slow burning axonal degeneration persists in such chronic MS lesions. One hypothesis for inflammation-independent axon degeneration is that the neuronal mitochondrial dysfunction leads to a lack of ATP in the axon and the loss of Na^+/K^+ ATPase activity, which is essential to maintain ionic balance in demyelinated axons (Waxman 2006). An increase of axonal calcium, exacerbated by mitochondrial dysfunction can activate a number of degenerative pathways. Evidence from MS postmortem tissue supports this mechanism. Degenerative axons are associated with co-localization of $Na_v1.6$ channels and the Na^+/Ca^{2+} exchanger.(Craner et al. 2004a)

Histological studies of human MS and animal model tissue also highlight the potential importance of complex IV activity over mitochondrial content. Degenerating axons in MS do not show the compensatory increase of axonal mitochondrial content nor complex IV activity but further studies suggest it is complex IV activity rather than mitochondrial content that is the more important factor.(Mahad et al. 2009) In the hemizygous PLP overexpression mouse model, axon degeneration was apparent in the light of an increase in mitochondrial content but no concurrent increase of complex IV activity.(Hogan et al. 2009) Our recent studies (unpublished) show that despite the fact that all classic demyelination animal models show an increase of axonal mitochondrial content upon demyelination, this is not always the case for complex IV activity. Following lysolecithin induced demyelination both mitochondrial content and complex IV activity increase within demyelinated axons. In contrast, we did not find a corresponding increase in complex IV activity within demyelinated axons in a number of EAE in mouse, rat and marmoset species, despite an increase in axonal mitochondrial content. Interestingly, the level of axonal of complex IV activity was significantly correlated with the amount of axonal damage within the demyelinated lesion, which was not the case for the amount of axonal mitochondrial content. Future studies should focus on the timing of complex IV loss and its relation to changes in axonal calcium level, mitochondrial reactive oxygen species production as well as mitochondrial membrane potential. Evidence from EAE suggests that the loss of axonal complex IV activity occurs relatively early compared with structural changes. The loss of complex IV activity in axonal mitochondria in EAE may precede axonal mitochondrial morphological changes.(Nikic et al. 2011) The mechanism of

complex IV activity loss may be nitration of complex IV subunit IV that occurs in EAE prior even to the arrival of inflammatory cells.(Qi et al. 2006)

The increased energy demand of demyelinated axons, highlighted by changes in the mitochondrial network, must be met by the neuronal cell body, the site of mitochondria generation (Figure 2). The dysfunction of the mitochondria in neuronal soma would therefore put the demyelinated axon at risk of degeneration.

Are the molecular changes intrinsic to neurons in progressive MS primary or secondary?

The nature of the autopsy tissue available in progressive MS makes the delineation of the time course of the neuron-specific irreversible mitochondrial injury challenging. Although biopsy material from early stage of MS has been used to define inflammation in the cortex such material is limited for more detailed analysis of mitochondria within neuronal cell bodies.(Lucchinetti et al. 2011) Inflammation is a potent inducer of reactive oxygen species and mitochondrial dysfunction in the CNS. Suppressing inflammation is an obvious strategy to limit or prevent mitochondrial dysfunction and facilitate the generation of healthy mitochondria in the surviving neurons. In progressive MS, however, our current view is that at least part of the molecular changes within neuronal cell bodies that compromise the mitochondrial respiratory chain are induced and irreversibly amplified within the CNS by the chronic inflammation and demyelination. Iron accumulation and increase in oxidative injury are likely to further amplify the mitochondrial damage over time. Chronic demyelination, as mentioned above, will be another factor that will trigger axonal mitochondrial injury independent of inflammation, as shown in *Shiverer* mice.(Joshi et al. 2015) Given the protracted clinical course of relapsing MS, the compromise of the neuronal compartment due to mitochondrial dysfunction may occur relatively early in progressive MS.

Therapeutic implication of the neuronal mitochondrial respiratory chain complex deficiency.

Immunomodulation has been a very successful strategy in relapsing MS and increasingly at least in a subset of patients with progressive MS. Drugs and monoclonal antibodies that target remyelination are currently being tested in clinical trials and appear to be a promising

strategy to protect axons in MS.(Franklin et al. 2012) We see the targeting of the neuronal bioenergetics, by improving mitochondrial function and dynamics, as an addition to the future combinatorial therapeutic strategies for progressive MS. In this respect, experimental disease models that reflect the inflammatory component as well as chronic demyelination and neuron specific mitochondrial injury are a much needed resource to both increase the understanding of the pathogenesis of progressive MS and to develop novel therapeutic strategies and agents.

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Figure legends

Figure 1

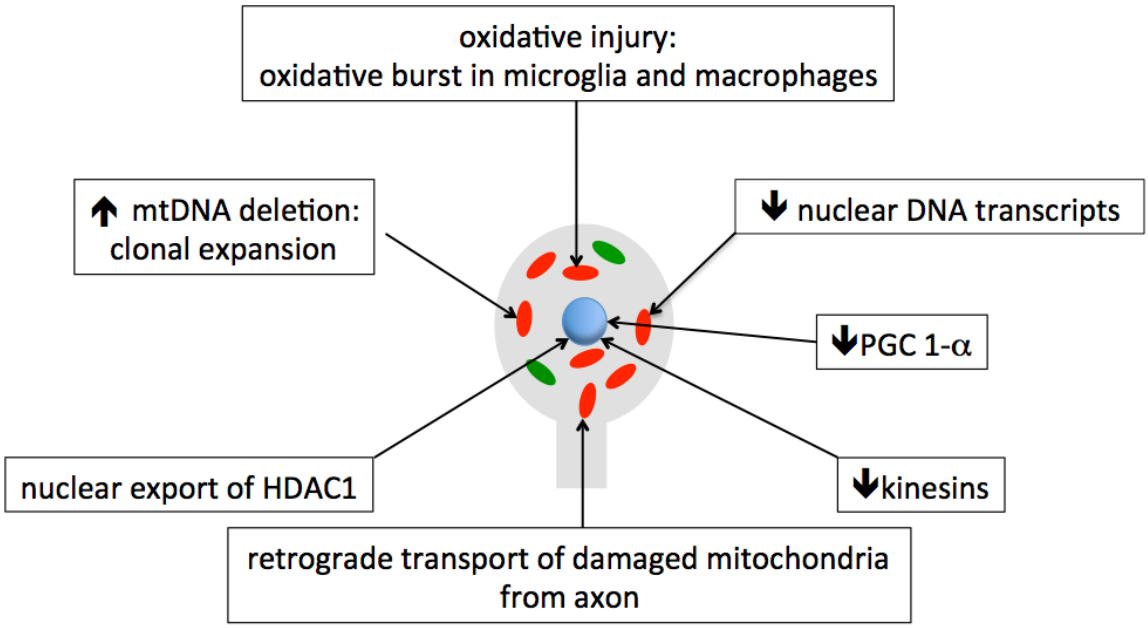

Figure 1. Molecular changes that are intrinsic to neuronal cell bodies in MS. A number of studies have now identified molecular changes, all o f which converge on mitochondria, in progressive MS. Dutta et al described a decrease in nuclear DNA encoded transcripts of mitochondrial respiratory chain complexes, which was reproduced by others. Witte et al proposed a decrease in PGC-1alpha as a possible cause of the decrease in nuclear encoded transcripts within neurons. Campbell et al identified clonally expanded mitochondrial DNA deletions (mtDNA) at high heteroplasmy level within single neurons in progressive MS cortex. Other studies reported molecular changes that impair anterograde transport of mitochondria, decrease in HDAC1 and kinesins, which are essential for replenishing the axon with healthy mitochondria in physiological conditions. In progressive MS, the respiratory chain complex deficiency means that the neuronal cell body is no longer capable of replenishing the axon with healthy mitochondria, particularly in long projection axons such as those in the corticospinal tracts (see Figure 2). Red: respiratory chain complex deficient mitochondria. Green: healthy mitochondria.

Figure 2

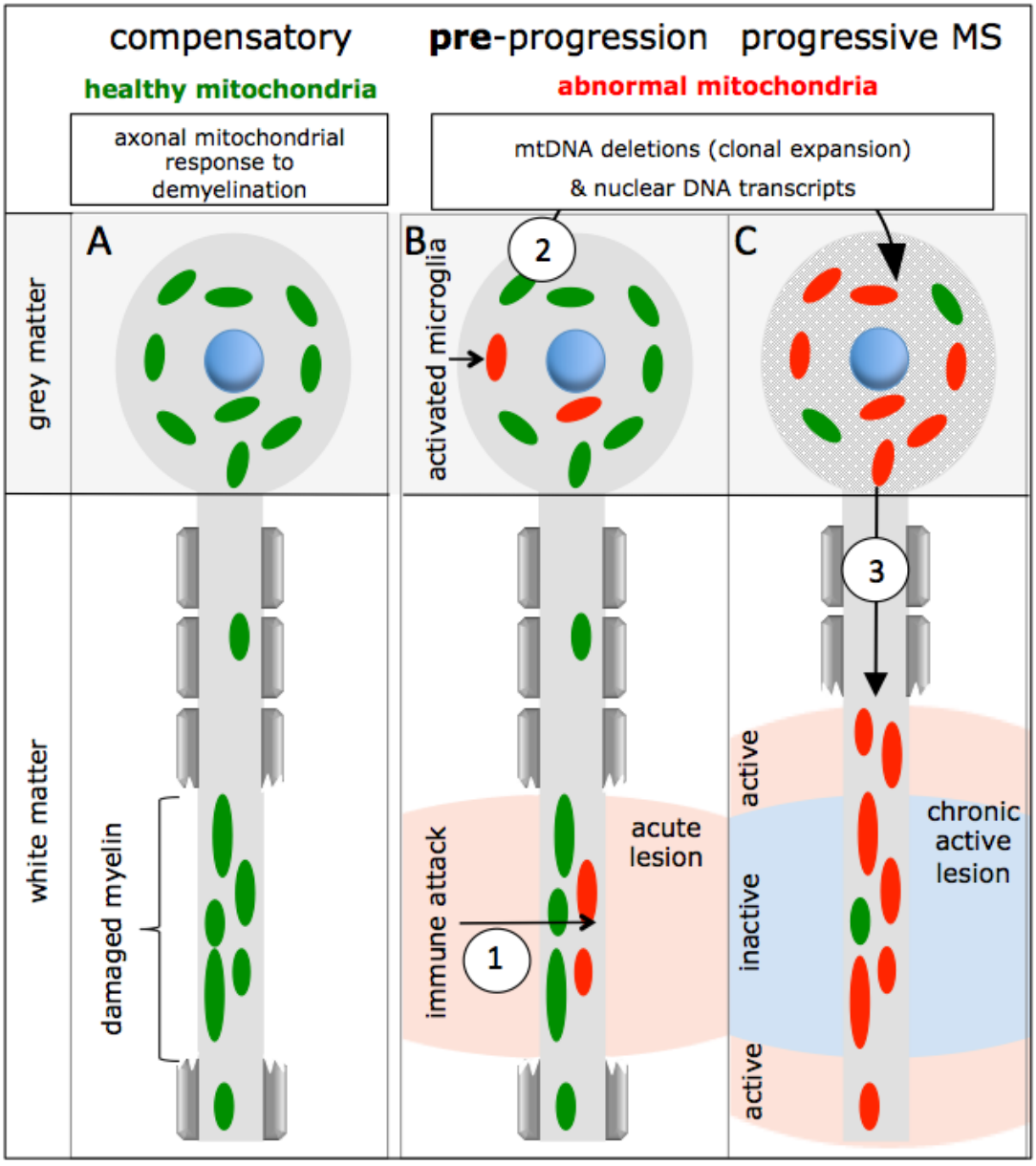

Figure 2. How neuronal mitochondria play a role in axon degeneration in progressive MS: A. We reported the gathering of functional mitochondria in the demyelinated axon in MS and upon non-autoimmune demyelination of wild type neurons in vivo, as previously published (Mahad et al. 2015). These mitochondrial changes (increased number, size, activity and speed of movement of mitochondria reported as “axonal mitochondrial response to demyelination”) protected the demyelinated axon since the inability to mount the axonal mitochondrial response to demyelination in mice lacking syntaphilin, an axon specific mitochondria docking protein, led to an excess of axon ovoids upon demyelination. B. During

pre-progressive stage of MS, inflammatory products injure mitochondria in multiple cell types including neurons and the oxidative injury to DNA leads to the formation of mtDNA deletions in both the white matter (1) and grey matter. Over time and with age abnormal mitochondria are amplified in neuronal cell bodies (2), for example, through clonal expansion of the inflammation induced mtDNA deletions and depleted nuclear DNA encoded mitochondrial transcripts. C. The resulting biochemical deficiency of the mitochondrial respiratory chain complexes or enzymes in neuronal cell bodies (indicated by the texture grey background) then act as a reservoir of abnormal mitochondria, which then undergo aberrant placement to the demyelinated axon and cause energy failure and increased reactive oxygen species production in the axon (3). In addition, any residual or active inflammation in progressive MS brain would further impair mitochondrial function particularly at the active edge of chronic active MS lesions. This forms a three step hypothesis (formation, amplification and displacement) for the role of mitochondria in axon degeneration in progressive MS. Arrows indicate the process through which the three step hypothesis leads to axon energy failure in progressive MS.

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